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NELLIE M. NAYLOR

DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF PURE SCIENCE, COLUMBIA UNIVERSITY

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INFLUENCE OF SOME ORGANIC COMPOUNDS UPON THE HYDROLYSIS OF STARCH BY SALIVARY AND PANCREATIC AMYLASES

Various organic compounds have been reported as influencing the activity of amylases in the digestion of starch. In 1893[1] and again in 1904[2], Effront investigated the effects of certain amines and amino acids upon the hydrolysis of starches by an infusion of malt extract. Glycine, alanine, leucine, glutamic acid, hippuric acid, creatine, creatinine, asparagine and aspartic acid were found to increase the activity of the amylase, while amides and aliphatic amines appeared to act as inhibitory agents. Ford and Guthrie,[3] using Lintner soluble starch with malt extract and with a purified malt amylase, studied the effect of asparagine, glycine and alanine on the starch digestion. They ascribe the apparent increase of activity in the presence of the amino acids to the amphoteric property of these compounds or to their effect in neutralizing some inhibitory impurity in the starch or enzyme solution.

Terroine and Weill[4] tested the influence of many of the amino acids on the saccharification of starch by pancreatic juice. They report an activating influence on the part of the amino acids tested, but they make no statement regarding the hydrogen-ion concentration, or regarding the purity of any of the materials used.

Desgrez and Moog[5] report an activating influence exerted by methyl, ethyl and trimethyl amine hydrochlorides, on the hydrolysis of starch by a glycerol extract of dry pancreas. As in the work reported by previous investigators, there is no indication of any tests for hydrogen-ion concentration or for purity of materials used. Nor is there any evidence that

[1] Effront, *Mon. Sci.*, **41,** 266 (1893).
[2] Effront, *ibid.*, **61,** 561 (1904).
[3] Ford and Guthrie, *J. Chem. Soc.*, **89,** 76 (1906).
[4] Terroine and Weill, *Compt. rend. soc. biol.*, **72,** 542 (1912).
[5] Desgrez and Moog, *Compt. rend.*, **172,** 553 (1921).

the influence of simple inorganic salts was provided for, so that so far as their data show, the activation may have been due simply to the effects of the substances as chlorides rather than to the organic radicals.

Rockwood[6] studied a large number of nitrogen compounds with regard to their influence on the hydrolysis of starch by saliva. Those compounds which seemed to increase the activity of the amylase were called auxo-amylases. It was reported that: (1) α-amino acids act as auxo-amylases, as evidenced by the effect of added glycine, tyrosine and aspartic acid; (2) anthranilic acid, and its *meta* and *para* isomers, in which the amino group is not alpha to the carboxyl group, were also reported to be auxo-amylases; but sulfanilic acid, in which the carboxyl group is replaced by the group, SO_2OH, was not; (3) the substitution of one hydrogen of the amino group by benzoyl, as in hippuric acid, did not destroy the activating properties; (4) amines of the methane series were reported to act as auxo-amylases; (5) amides were not auxo-amylases. The results of Rockwood's experiments are, however, not conclusive, since several factors now known to influence the activity of the amylase were not standardized and apparently not taken into account. As mentioned in connection with other work, there is no evidence that the influence of inorganic salts was provided for; also the hydrogen-ion concentration, determined as "neutral to litmus," would be only approximately known, and not necessarily that at which optimum activity of the amylase is obtained. Therefore the "activation" reported may be due to the influence of other factors, instead of to the effect of the organic groupings in the compounds tested.

In this Laboratory the work of testing the influence of amino acids on the hydrolysis of starch by amylases has been done under much more closely standardized conditions; the starch and all salts used were purified, the hydrogen-ion concentrations of all starch dispersions tested, either electrometrically or colorimetrically with standardized buffer mixtures, and the enzyme, either purified or natural, was always present in a starch paste "activated" by an optimum concentration of sodium chloride and sodium phosphate. Under these conditions, it has been determined[7] that neutralized aspartic acid and asparagine, glycine, alanine, phenylalanine and tyrosine increase the saccharogenic activity of saliva, pancreatin, and purified pancreatic and malt amylases. It was also determined[8] that glycine, phenylalanine, arginine and cystine increase the amyloclastic activity of purified pancreatic amylase, while histidine and tryptophane do not show this influence.

It was the purpose of this investigation to study the influence of certain organic compounds, containing typical groupings, on the hydrolysis of

[6] Rockwood, *J. Am. Chem. Soc.*, **39**, 2745 (1917).

[7] Sherman and Walker, (a) *ibid.*, **41**, 1866 (1919); (b) **43**, 2461 (1921).

[8] Sherman and Caldwell, *ibid.*, **43**, 2469 (1921).

starch by amylases, to see whether this might throw some light upon the problem of whether the favorable effect of amino acids is due to a direct activation attributable to their organic structure, as considered by Rockwood, or is due to the conservation of the enzyme, as brought out by previous work in this Laboratory,[7b,8] or due to both. It was planned to use several simple organic compounds, in which the influence of the carboxyl group alone, the amino group alone, and the carboxyl and amino groups in the same molecule, could be studied, and then to extend the investigation to the influence of other groupings, especially those present in the amino acids which have been tested in this Laboratory. Several of the compounds used in this work have been studied by other investigators, but since the results were inconclusive, as has been pointed out, the work has been repeated by the standardized method[7b] used in this Laboratory for testing the influence of the amino acids on the hydrolysis of starch by amylases. The compounds were chosen: (1) to contain the amino group in aniline sulfate and in methyl and ethyl amine hydrochlorides, the carboxyl group in benzoic acid, and the amide group in benzamide; (2) to show the effect of the position of the amino group by studying glycine, in which the amino group is *alpha* to the carboxyl group, and anthranilic acid in which the amino group is *ortho* to the carboxyl group; (3) to show the effect of substitution of the hydrogen of the amino group, as in hippuric acid; (4) to test the influence of indole and guanidine, and to compare their effect with that of the amino acids containing these groupings.

Materials Used

Lintner soluble starch was purified by repeated washings with distilled water and with thrice distilled water. The starch was air-dried and the moisture was determined. The acidity was determined by electrometric titration of a 1% starch dispersion containing the amounts of sodium chloride and disodium phosphate used for pancreatic amylase work.[8] All water used in making starch pastes and activating solutions, and in the final rinsing of glass ware, was distilled from alkaline permanganate, then from dil. phosphoric acid, through a block-tin condenser, and collected in Non-sol bottles, in which it was kept until used.

The sodium chloride and sodium phosphate used as activating agents were recrystallized twice from distilled, and once from thrice distilled water, air-dried, and analyzed for moisture. All of the organic substances employed were carefully purified and tested for purity.

Experimental Procedure

The equivalent of 10 g. of dry starch was weighed, mixed with cold water, poured into boiling water, and the mixture boiled for 3 minutes. This was cooled, made up to a volume of 250 cc., and allowed to settle. Twenty-five-cc. portions of this starch dispersion were introduced into 100cc. cylinders and the required amount of 0.01 *N* sodium hydroxide solution was added. The activating agents, sodium chloride and sodium phosphate, in amounts previously determined for the enzyme to be used[9] were added to the cylinders.

[9] Sherman and Kendall, *J. Am. Chem Soc.*, **32**, 1087 (1910).

The substances to be tested in this work were in most cases difficultly soluble, and more or less subject to hydrolysis. The amount to be used for several cylinders was weighed out, water and the small quantity of sodium hydroxide (0.01 *N* or 0.02 *N*) or hydrochloric acid (0.01 *N*) required for neutralization were added to the substance, and carefully warmed not above 40°, until the substance was dissolved. This solution was cooled, and made up to a definite volume, and portions to represent the desired quantity of the substance were added to the cylinders, from a buret. The contents of each cylinder was then made up to a total volume of 100 cc., stirred, and the cylinders were placed in a thermostat regulated at 40±0.01°. While the cylinders were attaining the temperature of the surrounding water, the enzyme solution was prepared, and introduced into clean, dry flasks. The activated starch dispersion at 40° was added to the enzyme, the whole thoroughly mixed and allowed to react for exactly 30 minutes, the flasks being kept at constant temperature. The effect of light was excluded by working in a north room with window shades drawn, and avoiding the use of any artificial light during digestion. The reaction was stopped by the introduction of Fehling solution, and the flasks were placed in a boiling water bath for 15 minutes. The amount of cuprous oxide formed by the reducing sugar present was determined.

Since sodium sulfate would be present in every test in which neutralized aniline sulfate was used, any difference in the activity of the enzyme might be due to the effect of the aniline or to the sodium sulfate. When sodium sulfate was tested in amounts corresponding to those which would be formed in the solutions containing neutralized aniline sulfate, it was found to have no influence on the activity of the amylases used.

Since earlier work[10] has shown that the optimum activity of pancreatic amylase is obtained for only a small range of hydrogen-ion concentration, it is necessary to deter-

TABLE I

LOG C_{H^+} IN MOLES PER LITER FOUND IN DIGESTION MIXTURES USED

0.01 *M* solution added except with benzoic acid and benzamide, of which 50 mg. each was added

Starch Paste (activated)	Log C_{H^+} (electrometric)	Log C_{H^+} (colorimetric)
No substance added	−6.94	−6.93
Benzoic acid	−6.96	−6.93
Benzamide	−6.90	−6.93
Aniline[a]	−6.87	−6.93
Hippuric acid	....	−6.93
Anthranilic acid	....	−6.93
Methyl amine hydrochloride	−6.90	−6.93
Ethyl amine hydrochloride	−6.89	−6.93
Indole	−6.87	−6.93
Guanidine hydrochloride	−6.88	−6.93
Tryptophane	−6.96	−6.93
Alanine	−6.86	−6.93

[a] Aniline sulfate, calculated to the desired amount of aniline, was used in all cases.

mine accurately the values for each digestion mixture used. These determinations were made by electrometric titration (if possible) of the substance to be tested, in a buffered starch dispersion; then in each "set" the hydrogen-ion concentration was checked by a colorimetric test of the starch dispersion actually used in digestion, or that of a separate cylinder made up exactly like the one used. In all colorimetric tests the

[10] Sherman, Thomas and Baldwin, *J. Am. Chem. Soc.*, **41**, 231 (1919).

color comparison was made with a standardized buffer solution,[11] using bromothymol blue as indicator, range P_H 6.0 to 7.6. The results of these hydrogen-ion determinations are given in Table I.

In order to prove that hydrolysis of the substances tested did not change the hydrogen-ion concentration of the mixtures during digestion, colorimetric or electrometric tests were made of the starch dispersions, before and after digestion. In all cases, where solutions were buffered with sodium phosphate, the hydrogen-ion concentration was found to be constant throughout the experiment.

Data of Typical Experiments

Table II shows the influence of equimolar quantities of methyl and ethyl amine hydrochlorides, aniline, anthranilic acid, hippuric acid and glycine, when tested in the presence of activating salts, sodium chloride and sodium phosphate, and when tested in the absence of these salts.

TABLE II

INFLUENCE OF EQUIMOLAR AMOUNTS OF CERTAIN ORGANIC SUBSTANCES ON THE HYDROLYSIS OF STARCH BY SALIVA

Added material	In presence of salts	In absence of salts	
	Cc. of pure saliva per 100 cc. of starch dispersion		
	0.4	0.4	0.8
0.01 *M*	Cuprous oxide Mg.	Cuprous oxide Mg.	Cuprous oxide Mg.
None	294	2.6	..
Methyl amine hydrochloride	297	281.0	..
Ethyl amine hydrochloride	293	285.0	..
None	208	0.5	4.6
Aniline	202	3.0	25.0
Anthranilic acid	191	6.0	15.0
None	197	2.0	4.5
Hippuric acid	180	1.0	22.0
Glycine	209	0.3	4.3

This experimental work shows that very small amounts of reducing sugar are obtained by the action of saliva on a pure starch dispersion, in the absence of added electrolytes, as has also been found to be true in the case of the pancreatic amylase, even when tested in the form of commercial pancreatin.[12] The presence of 0.01 *M* ethyl and methyl amine hydrochlorides, in the media in which no other salts are added, activates the enzyme to such an extent that almost as much reducing sugar is obtained as in the presence of sodium chloride and sodium phosphate. Since aniline, tested as sulfate, does not show this effect, the influence exerted by methyl and ethyl amine hydrochlorides cannot properly be interpreted as showing any specific effect of the amino group, but is probably due to the favorable

[11] W. M. Clark, "Determination of Hydrogen Ions," Williams and Wilkins Co., 1920, p. 81.

[12] Sherman and Schlesinger, *J. Am. Chem. Soc.*, **34**, 1104 (1912); **37**, 1305 (1915).

influence upon the amylase of the chloride ions thus introduced into the digestion mixture.[9]

Anthranilic acid, hippuric acid and glycine tested in the absence of inorganic salts, showed no influence on the rate of digestion of starch when 0.4 cc. of pure saliva per 100 cc. of starch dispersion was used. However, when the concentration of enzyme was doubled in the starch dispersions containing these substances, no inorganic salts being added, thus making the conditions comparable with the experiments described by Rockwood, an activating influence was obtained. This activation may be attributed to the presence of a larger amount of electrolyte in the increased volume of saliva used, rather than to the effect of the substances added. This view is supported by the fact that when anthranilic acid, hippuric acid and aniline are tested in the presence of sodium chloride and sodium phosphate, they show no "activating" influence. These experiments confirm the statements already made, that tests for the influence of any substances on the digestion of starch by saliva or by pancreatic amylase must be made in the presence of inorganic salts;[9] and indicate that the "activation" reported by Rockwood and other investigators as attributable to organic structure is misleading, and is probably due to the influence of the added substances upon hydrogen-ion or electrolyte concentration rather than to any specific effect of the organic groups.

In order to determine whether the organic groups discussed by Rockwood have activating effects upon salivary and pancreatic amylases when

TABLE III

INFLUENCE OF BENZOIC ACID, ANILINE SULFATE, BENZAMIDE, ANTHRANILIC ACID AND HIPPURIC ACID ON THE HYDROLYSIS OF STARCH BY SALIVA AND BY PURIFIED PANCREATIC AMYLASE

Material added		Saliva	Pancreatic amylase
	Mg.	Cuprous oxide Mg.	Cuprous oxide Mg.
None	...	327	298
Benzoic acid	50	324	274
Benzoic acid	100	321	272
None	...	331	282
Hippuric acid	50	322	261
Hippuric acid	100	314	254
None	...	218	217
Aniline	50	217	206
Aniline	100	225	203
None	...	296	312
Benzamide	50	289	310
Benzamide	100	289	308
None	...	331	317
Anthranilic acid	50	313	305
Anthranilic acid	100	319	305

these are tested in the presence of the usual "activating" salts, a series of experiments was carried out with saliva and pancreatic amylase tested

in the presence and absence of benzoic acid, hippuric acid, aniline sulfate, benzamide or anthranilic acid.

The technique of these experiments was the same as has already been described, the mixtures always being "activated" by chloride and phosphate and made up to the correct hydrogen-ion concentration for the enzyme to be used. Typical results are shown in Table III. The results obtained with benzoic acid and with aniline sulfate (Table III) and methyl and ethyl amine hydrochlorides (Table II) on the hydrolysis of starch by amylases, are taken as typical of the influence of the carboxyl and amino groups, when tested under conditions suitable to the normal activity of the enzyme. Since no favorable effect is shown by any of these compounds, the presence of the carboxyl group alone or the amino group alone does not account for the "activation" of the amylases by amino acids. Benzamide shows no effect on the activity of the amylases used. Glycine, as well as most other natural amino acids, has been shown to give definite "activation" of the amylases in the digestion of starch,[7b] while anthranilic acid, containing both the amino and carboxyl groups does not activate.

Since in these experiments only the α-amino acids, such as glycine, and other products of protein hydrolysis have been found to increase the activity of the amylases, it is plain that neither the amino nor the carboxyl group alone, nor the presence of both in the same molecule is sufficient to induce any "activating" influence upon the amylases when present in a substrate solution containing proper amounts of simple electrolytes.

One may, therefore, conceive either that the "activating" effects of amino acids, like glycine, are due to their structural configuration in that they contain amino radicals in the α position to the carboxyl group, or that their favorable influence is due to the conservation of the enzyme through diminution of its hydrolytic destruction in the water solutions in which it acts.[7b] It has not been feasible to test α-amino acids known not to be products of protein hydrolysis because our knowledge of the hydrolytic products of the proteins is not yet sufficiently complete. It might, perhaps, be expected that if true "activation" can be attributed to α-amino acid structure, *per se*, it should not be entirely lost in a derivative such as hippuric acid (benzoyl glycine). Hippuric acid, however, shows no "activating" effect on the digestion of starch by saliva and purified pancreatic amylase.

It was thought best to determine whether this failure to "activate" the enzyme might be due to the hydrolysis of the hippuric acid during digestion, forming glycine and benzoic acid. By a colorimetric test, using standardized buffer solutions,[13] and bromothymol blue as indicator, it was found that the hydrogen-ion concentration was the same before and after

[13] Ref. 11, p. 76.

digestion and, therefore, that hydrolysis of hippuric acid did not occur to any appreciable extent. Table IV shows a typical experiment in which the influence of glycine alone, of benzoic acid alone, of a mixture of equal weights of glycine and benzoic acid, and of equimolar amounts of each, is tested.

TABLE IV

COMPARISON OF THE INFLUENCE OF BENZOIC ACID PLUS GLYCINE ON THE HYDROLYSIS OF STARCH BY PURIFIED PANCREATIC AMYLASE

Material added Mg.	Cuprous oxide Mg.
None	288
Benzoic acid 50	280
Glycine 50	331
Benzoic acid 50 + glycine 50	335
Benzoic acid 0.0066 *M*, + glycine 0.0066 *M*	326

This experiment shows the usual "activation" with glycine, independent of the presence of the benzoic acid.

The effect of indole and guanidine on the hydrolysis of starch by purified pancreatic amylase, and the influence of these groups in the amino acids, were next tested. It was found that guanidine, like arginine, reacts with Fehling solution so that a test upon saccharogenic activity could not be made. The influence of these substances on the amyloclastic activity of the enzyme was studied, instead, by a method based on that of Wohlgemuth,[14] and used in previous work in this Laboratory.[15] The results indicate that guanidine has no effect on the amyloclastic activity of the enzyme, while indole shows an inhibitory effect. This accords with the observation that arginine has a favorable effect upon the amyloclastic action of the enzyme while tryptophane has not.[8] In view of these experiments, one might reason that the indifferent behavior of tryptophane, compared with the activating influence of most of the other amino acids, is explainable on the hypothesis that the favorable effect of its alanine group is offset by the inhibitory influence of the indole radical. However, the explanation that tryptophane may be so bound in the enzyme molecule that it is not liberated by hydrolysis until after the amyloclastic activity of the enzyme has been injured, seems more consistent, when studied in the light of further investigation. When a comparison was made of the influence of alanine, phenylalanine and tryptophane on the saccharogenic activity of the amylase, the result showed that tryptophane acts like most of the other amino acids in increasing the saccharogenic activity of the enzyme. The results of a typical comparison are given in Table V.

[14] Wohlgemuth, *Biochem. Z.*, **9**, 1 (1908).

[15] Sherman and Thomas, *J. Am. Chem. Soc.*, **37**, 634 (1915). Ref. 8.

TABLE V

INFLUENCE OF ALANINE, PHENYLALANINE, TRYPTOPHANE AND INDOLE ON THE HYDROLYSIS OF STARCH BY PURIFIED PANCREATIC AMYLASE

Material added 0.005 *M*	Cuprous oxide Mg.	Material added 0.005 *M*	Cuprous oxide Mg.
None.....	300		
Alanine.....	339	Tryptophane.....	338
Phenylalanine.....	341	Indole.....	275

Since consistent "activation" is obtained with tryptophane as with other amino acids tested, upon the saccharogenic property of the amylase, it is evident that in this case the added substance affects the amyloclastic and saccharogenic activities differently. With the other amino acids here tested, the influence has been the same towards the two properties of the enzyme; but tryptophane is not unique in augmenting the saccharogenic but not the amyloclastic activity, for in other experiments in this Laboratory the same has been found with respect to lysine.[16]

This effect is not due to hydrogen-ion concentration, since this was constant in the digestion mixtures used in all experiments, as has been stated before, and since previous work[17] has shown that, for optimum activity of the amyloclastic and saccharogenic properties of pancreatic amylase, the range of hydrogen-ion concentration is the same. However, since the enzyme molecule is in all probability of a protein nature, the tryptophane may be so bound in the molecule that it would not be liberated until after the amyloclastic activity of the enzyme was lost and, therefore, any added tryptophane would not affect the amyloclastic property, but still might protect the enzyme from further hydrolytic changes whereby its saccharogenic activity would be affected.

Summary

The favorable effect reported by Rockwood to be exerted by several types of organic compounds upon the activity of amylolytic enzymes, in consequence of which he applied the term auxo-amylases to these compounds, appears to have been due in most if not all cases, other than those of natural amino acids, to hydrogen-ion or salt effects, rather than to the organic structure of the compounds.

Tested upon salivary or pancreatic amylase in the presence of favorable concentrations of chloride, phosphate and hydrogen ions, methyl and ethyl amine hydrochlorides, aniline sulfate, benzoic acid, benzamide, anthranilic acid and hippuric acid failed to show any favorable effect upon the activity of the enzyme. Hence, it appears probable that none of the types of compounds illustrated by these substances has any activating influence upon salivary or pancreatic amylase which can properly be attributed to their organic structure.

[16] Sherman and Caldwell, *J. Am. Chem. Soc.*, **44**, 2926 (1922).

[17] Sherman and Schlesinger, *ibid.*, **35**, 1784 (1913).

Previous findings regarding the favorable influence of several amino acids resulting from protein hydrolysis have been confirmed and extended. This influence may be attributed either to a direct "activating" effect dependent upon the structural nature of these substances as α-amino acids, or to conservation of the enzyme by retarding its hydrolysis. While the hypothesis of direct "activation" exerted by α-amino compounds as such is not disproved, the results of tests with hippuric acid fail to give it any support. The results obtained in this investigation can all be explained on the basis of the conservation hypothesis alone.

VITA

Nellie M. Naylor was born at Clear Lake, Iowa, March 20, 1885. She prepared for college at the Clear Lake High School, and entered Iowa State College in 1902, taking two years of collegiate work there and later, two years at the State University. She received the degeree of Bachelor of Arts from Iowa State University in 1908. She was an Assistant in Chemistry at Iowa State College from 1909 to 1911, and an Instructor in Chemistry from 1911 to 1920. She was a graduate student at the University of Chicago during the summers of 1910, 1911 and 1919, and received the degree of Master of Science at Iowa State College in June, 1918. She has been a graduate student in the School of Pure Science Columbia University during the academic years 1920–1921 and 1921–1922.

She was co-author with Dr. R. R. Renshaw of a paper entitled "Dyes containing the Furane Cycle," published in the *Journal of the American Chemical Society* **44,** 862 (1922).

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